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Titration microcalorimetry

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Key features of an isothermal titration microcalorimeter (ITC) are described together with a general equation which forms the basis of the analysis of the calorimetric results. Recent applications of titration microcalorimetry to four subject areas concerned with the properties of aqueous solutions are reviewed. The four subjects covered are (i) pairwise enthalpic solute–solute interaction parameters in aqueous solution, (ii) deaggregation of micelles formed by ionic surfactants, (iii) protein–ligand interactions and (iv) adsorption of adsorbates on polymeric adsorbents.

1 Introduction

Calorimetry has made an enormous contribution to our understanding of chemical reactions and the properties of chemical substances. Many different designs for calorimeters have been described with specific tasks in mind,[1,2] each type of calorimeter requiring a characteristic method of data analysis. This review is concerned with a particular type of calorimetry based on a titration microcalorimeter[3–6] with particular reference to the calorimeter manufactured by MicroCal Inc. (USA). This technique takes advantage of modern electronics linked to computer-based control and computer-based data analysis. We illustrate the general features of this technique by reference to the calorimeter described in ref. 3. This calorimeter was originally designed to quantify enzyme–substrate interactions. Hence, only minimal amounts of chemical substances were required exploiting the high sensitivity of the calorimeter.[4] The high sensitivity has meant that the range of applications has broadened considerably. We consider the impact that titration microcalorimetry has and can have on a range of interesting physicochemical problems including micelle deaggregation and adsorption by polymers in aqueous solution. We describe a general treatment of the thermodynamics of titration microcalorimetry. We indicate how this general treatment is reformulated for analysis of calorimetric results for different types of systems.

2 Isothermal titration microcalorimeter (ITC)

A precision matched pair of sample and reference cells of accurately known volume (*ca.* 1.5 cm^3) are held within an adiabatic jacket, Fig. 1. A microsyringe under computer control injects at predetermined time intervals a small volume (*e.g.* 5×10^{-6} dm^3) of a solution containing δn_j^0 moles of chemical substance-j into a solution of known composition held in the sample cell. A change in the extent of chemical reaction,[7,8] ξ, (or simply molecular organisation, see below) spontaneously occurs in the sample cell.[9]

The calorimeter senses the accompanying heat q which can be either exothermic or endothermic. The calorimeter operates in a differential mode. The reference cell contains a solution which matches the solution in the sample cell at the start of the experiment (*e.g.* pH, buffer system, salt concentrations) except for the reacting substance. The reference cell is heated at a very slow rate using a small constant electric power. The control system (Fig. 1) monitors the temperatures of both reference and sample cells adjusting the electrical power to the heaters of the sample cell to maintain the two cells at the same temperature (actually at a very small constant temperature difference). If injection of a small aliquot of solution from the stirred syringe is exothermic, the control system stops heating the sample cell until the temperature of the reference cell has caught up with the temperature of the sample cell. The recorded quantity is the rate of heating of the sample cell over the time required to bring sample and reference cells back on a common temperature ramp. The signal from the control unit is integrated to yield the heat q associated with a given injected aliquot from the syringe into the sample cell. These heats are of the order of milli-calories and the temperatures of the cells change by just a few milli-Kelvin. Thus, to all intents and purposes the calorimeter operates under isothermal and isobaric (equal to ambient pressure) conditions. The calorimeter is calibrated by a small pulse of electrical heating of the sample cell. The information recorded during a given experiment is the heat q per mole of injected substance for a series, say, of 25 injections. The calorimeter can be operated over the range from 0 to 80 Celsius which means that, for example, standard heat capacities of reaction can be calculated from the dependence of standard enthalpies of reaction

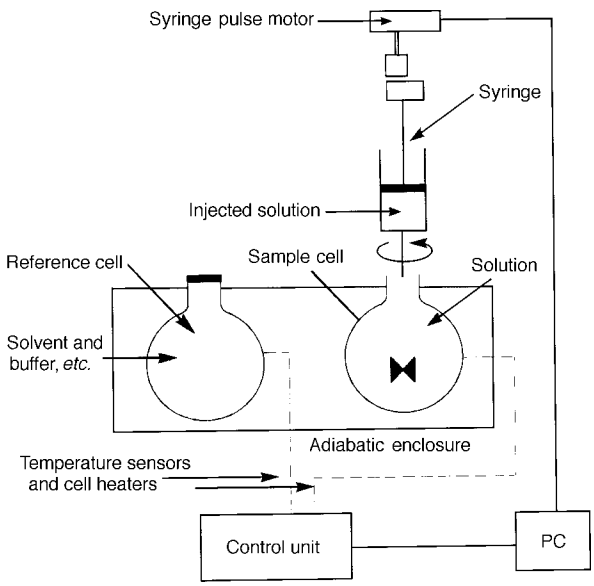


Fig. 1 The titration microcalorimeter showing reference cell and sample cell; the latter is fitted with a stirred syringe for injecting aliquots of a solution into the solution held in the sample cell

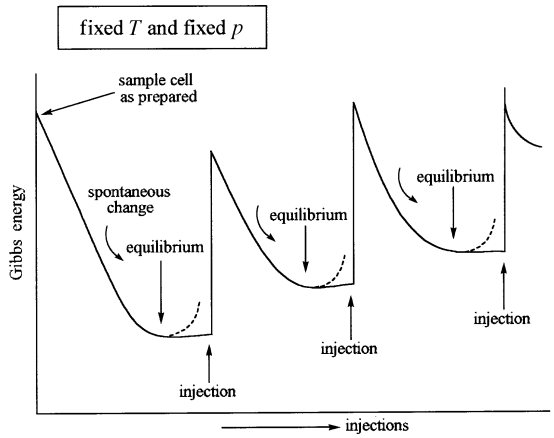


Fig. 2 Gibbs energies (at fixed temperature and fixed pressure) of the solution in the sample cell showing spontaneous changes and approach to minima in Gibbs energy G following injection of aliquots of solution from the syringe

on temperature. There is merit in using aqueous solutions because the heat capacity of water (and aqueous solutions) per unit volume is larger than most other liquids. Consequently, this large heat capacity smooths out the impact of instrumental noise.

The key point in the operation of the ITC is that heat q is the reporter of chemical processes occurring in the sample cell. Molecular interactions or reorganisations which are athermal are transparent to the observer. Each injection of an aliquot containing chemical substance-j is characterised by two quantities, q and the amount of δn_j^0. Therefore, in order to interpret this set of calorimetric results, a model is required for the chemical reaction or processes occurring in the sample cell consequent upon injection of an aliquot. The pattern formed by the dependence of the ratio $(q/\delta n_j^0)$ on injection number offers a sensitive test of the chosen model.

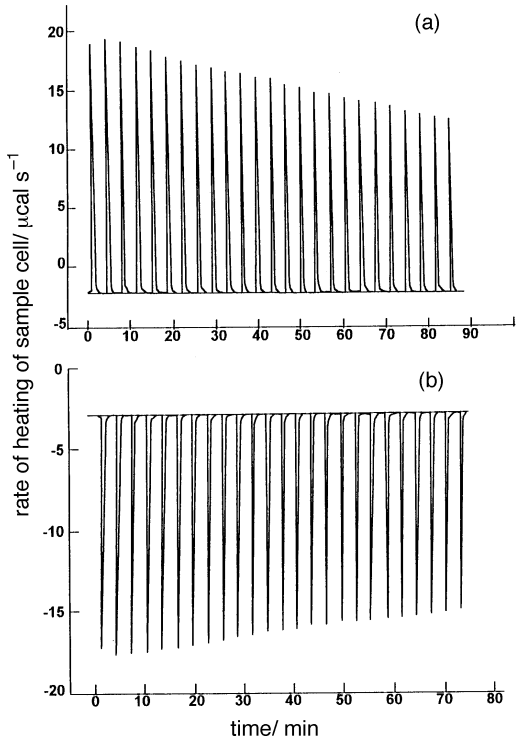


Fig. 3 Directly recorded[15,19] titration calorimetric plots for injection in water (l) of (a) urea (0.828 mol kg^{-1} in syringe) and (b) monoethylurea(aq) (0.796 mol kg^{-1} in syringe). Both plots show rate of heating of sample cell as a function of time.

Analysis of the calorimetric data is based on the condition that following each injection of δn_j^0 moles of substance-j from the syringe, the solution in the sample cell undergoes spontaneous chemical reaction in order to track through a series of thermodynamic equilibrium states. In other words, heat q is associated with a change in composition or organisation of the sample cell from prior to injection of an aliquot in one equilibrium state to after injection in another equilibrium state. Because these spontaneous changes occur at effectively constant temperature and constant pressure, the key thermodynamic potential function is the Gibbs energy. The changes taking place over a series of injections (Fig. 2) lower the Gibbs energy of the solution in the sample cell. Crucially, therefore, the time step between each injection must be sufficiently long compared with the half-life of all processes in the solution to allow the equilibrium states to be reached. This condition is confirmed by a small length of baseline between the pulses of heat recorded as a function of injection number. The plot generated by the dependence of $(q/\delta n_j^0)$ on, for example, the injection number is conveniently called an enthalpogram; see, for example, Fig. 3.

3 Thermodynamics

Within the sample cell, spontaneous changes in composition or organisation lower the Gibbs energy of the solution in the sample cell. The underlying assumption is that for a given solution across the whole range of possible compositions or organisations ξ (at fixed temperature and pressure), the minimum in Gibbs energy at equilibrium, is unique[10] and that the equilibrium state is stable.[9] Nevertheless, thermodynamics does not define *a priori* the dependence of enthalpy H on ξ about the equilibrium ξ^{eq} where G is a minimum. In other words, enthalpy H is not at an extremum at equilibrium.

In general terms, the state extensive variable H is defined by the three independent variables,[9] T, p and ξ, eqn. (1).

$$H = H[T, p, \xi] \tag{1}$$

The complete differential of eqn. (1) produces an equation for the differential change in enthalpy at fixed temperature and pressure, eqn. (2).

$$\mathrm{d}H = \left(\frac{\partial H}{\partial \xi}\right)_{T,p} \mathrm{d}\xi \tag{2}$$

Then at constant pressure, the first law of thermodynamics requires that the heat q accompanying the change in equilibrium composition of the solution in the sample cell from that after injection I to that after injection $I + 1$ (see Fig. 2) can be expressed as follows.

$$[q]_I^{I+1} = \left[\left(\frac{\partial H}{\partial \xi}\right)_{T,p} \mathrm{d}\xi\right]_I^{I+1} \tag{3}$$

In the titration experiment the change in composition $\mathrm{d}\xi$ is a result of injecting δn_j^0 moles of chemical substances-j. Then eqn. (3) can be written in the following form.

$$\left[\frac{q}{\delta n_j^0}\right]_I^{I+1} = \left[\left(\frac{\partial H}{\partial \xi}\right)_{T,p} \frac{\mathrm{d}\xi}{\mathrm{d}n_j^0}\right]_I^{I+1} \tag{4}$$

Eqn. (4) is the key equation for titration microcalorimetry. The left-hand side of eqn. (4) is the recorded quantity as a function of I and is given, according to the right-hand side, by the product of two terms, neither of which is known *a priori*. Therefore, in order to interpret the titration calorimetric data we require a model for the chemical reactions or molecular reorganisation occurring in the sample cell. The analysis is based on a consideration of the pattern formed by plots of

$$\left[\frac{q}{\delta n_j^0}\right]_I^{I+1}$$

as a function of either I or the total concentration of substance-j in the sample cell: the enthalpogram.

4 Pairwise enthalpic solute–solute interaction parameters

We consider the results of an experiment in which the sample cell contains initially water whereas the syringe contains a dilute aqueous solution of a neutral solute, chemical substance-j, *e.g.* urea. Then each injection of an aliquot of the solution of solute-j into the sample cell is a simple dilution. Consequently, the mean distance between solute molecules increases on injection into the sample cell. For example, according to the simple model described by Robinson and Stokes,[11] the mean distance apart for a simple solute-j increases from 5.4 nm at a concentration of 0.1 mol dm^{-3} to 25.5 nm at 1×10^{-3} mol dm^{-3}. In the experiment indicated above, the solution is dramatically diluted, effectively to infinite dilution when a dilute solution of solute-j is placed in the syringe.

In general terms, the enthalpy of an aqueous solution at fixed temperature and pressure, H(aq) prepared using n_1 moles of water and n_j moles of chemical substance-j is given by eqn. (5).

$$H(\text{aq}) = n_1 H_1^*(l) + n_j \phi(H_j) \quad (5)$$

In eqn. (5), $H_1^*(l)$ is the molar enthalpy of pure water at the same temperature and pressure whereas $\phi(H_j)$ is the apparent molar enthalpy of the solute. If the properties of this aqueous solution were ideal in a thermodynamic sense, eqn. (5) takes the following form.

$$H(\text{aq; id}) = n_1 H_1^*(l) + n_j \phi(H_j)^\infty \quad (6)$$

Here $\phi(H_j)^\infty$ is the limiting (infinite dilution) apparent molar enthalpy of solute-j in aqueous solution. Thus,

$$\text{limit}(m_j \to 0) H_j(\text{aq}) = H_j^\infty(\text{aq}) = \phi(H_j)^\infty \quad (7)$$

The definition in eqn. (7) applies at all temperatures and pressures; m_j is the molality of the solute. From eqn. (7) and (6),

$$H(\text{aq}) - H(\text{aq; id}) = n_j[\phi(H_j) - \phi(H_j)^\infty] \quad (8)$$

By definition the apparent relative molar enthalpy of the solute, $\phi(L_j)$, is given by eqn. (9).

$$\phi(L_j) = \phi(H_j) - \phi(H_j)^\infty \quad (9)$$

Then for a solution prepared using 1 kg of water,

$$H^E(\text{aq}) = H(\text{aq; } w_1 = 1 \text{ kg}) - H(\text{aq; } w_1 = 1 \text{ kg; id})$$
$$= m_j \phi(L_j) \quad (10)$$

Here H^E(aq) is the excess enthalpy for a solution prepared using 1 kg of water. Further, H^E(aq) is a member of a family of excess properties of such solutions which includes the excess Gibbs energy, G^E. For dilute solutions of neutral solutes, these excess thermodynamic properties are quadratic functions of the molality, m_j; see, for example, discussions in ref. 12–14. Thus, for dilute solutions of neutral solutes, H^E(aq) is expressed in terms of a pairwise enthalpic solute–solute interaction parameter characteristic of solute-j, $m^0 = 1$ mol kg^{-1}.

$$H^E = h_{jj}(m_j/m^0)^2 \quad (11)$$

Therefore, using eqn. (10)

$$\phi(L_j) = h_{jj}(m^0)^{-2} m_j \quad (12)$$

Combination of eqn. (12) and (4) shows that the calorimetrically derived ratio, $q/\delta n_j^0$ is related[15] to h_{jj} and the molality of solute in the syringe m_j^{sy} using eqn. (13).

$$q/\delta n_j^0 = -\phi(L_j)^{sy} = -h_{jj}(m^0)^{-2} m_j^{sy} \quad (13)$$

The significance of the quantity h_{jj} (and the corresponding Gibbs energy, g_{jj} and entropy, s_{jj}, pairwise interaction parameters) is understood in terms of a description of aqueous solutions advanced by Gurney.[16] Each solute molecule is surrounded by a cosphere of solvent molecules whose organisation and water–water interactions differ from those in pure water at the same temperature and pressure. Two broad classes of solutes are identified: (i) hydrophilic solutes where there is strong solute–solvent interaction (*e.g.* hydrogen bonding), and (ii) hydrophobic solutes in which water–water interactions in the cospheres are significantly stronger than solute–water interactions. In real solutions these cospheres interact[17,18] leading to deviations in the properties of solutes from ideal. Titration microcalorimetry offers a direct method for probing the enthalpic h_{jj} parameters. Indeed, the contrast between two solutes having only a slight difference in molecular structure is often striking. An example of this contrast is shown (Fig. 3) by the results for aqueous solutions containing urea and monoethylurea.

The results for urea(aq) show a series of endothermic peaks accompanying an increase in solute–solute distance. For monoethylurea, a series of exothermic peaks are recorded signalling that (in the reverse sense) mutual approach of two monoethylurea molecules in aqueous solution is endothermic. Clearly, replacement of a hydrogen in urea by an ethyl group has a dramatic effect on solute–solute interactions, a complete reversal of sign for h_{jj}. The pattern is consistent with the general model that hydrophobic pairwise interactions between monoethylurea molecules in aqueous solution are entropy driven against an otherwise enthalpic interaction. The results in Fig. 3 support this idea although we recognise that the term 'hydrophobic bond' arouses intense debate.[20]

The analysis can be taken a stage further in which the pairwise solute–solute interaction parameters are resolved into group interaction parameters.[21,22] The SWAG (≡Savage–Wood additivity of group interactions) parameter has attracted attention[22,23] together with its extension to analysis of kinetic data.[14,24–27] In an analogous fashion the pairwise enthalpic parameters for alcohols and sugars can be correlated[28] in terms of the number of OH-groups and the stereochemistry of the solutes. In other words, ITC has the potential for examining structure–interaction parameters for solutes in solution.

5 Ionic surfactants

The subject matter in this section develops from the ideas discussed in the previous section. Rather than a simple increase in solute–solute intermolecular distances, however, the injected aliquot contains aggregates of solute molecules in aqueous solution which fall apart on being injected into the sample cell containing initially water. In particular, we consider cases where the injected aliquot contains an ionic surfactant at a concentration above the critical micellar concentration,[29–32] c.m.c. We illustrate this application of ITC by reference to surfactants formed by alkyltrimethylammonium bromides and alkylpyridinium iodides. At the start of each experiment the syringe contains a solution at a concentration of a factor of *ca.* 20 greater than the c.m.c. We envisage that the micelles have a hydrophobic core, the ionic head groups being hydrated but binding in the double layer a percentage of the total counter-ions in solution. This model is the classic Hartley model although we recognise that other models for micelles have been discussed,[33] *e.g.* the Menger model.[34] In the case of the ionic surfactant, hexadecyltrimethylammonium bromide (CTAB), micelle formation is exothermic and so in the ITC experiment a series of endothermic pulses are recorded characterising micelle deaggregation. In the Utopian experiment (*i.e.* thermodynamically ideal solutions in both syringe and sample cell), a series of equal

intensity pulses would be recorded (Fig. 4). This pattern continues until the concentration of surfactant in the sample cell reaches and then passes the c.m.c. When the latter condition is reached the ITC records the heat accompanying the injection of a micellar solution into a micellar solution. Again for ideal solutions the recorded heat is zero. Thus, the break in pattern occurs at the c.m.c. The latter is pinpointed by a van Os plot.[35] The sum

$$\sum_{I=1}^{I}\left(\frac{q}{\delta n_j^0}\right)$$

plotted against the concentration of surfactant in the sample cell generates two straight lines, the concentration at the intersection point corresponding to the c.m.c.[35] In other words, ITC yields both enthalpies and Gibbs energies of micelle formation. The latter quantity is obtained from the c.m.c. although the relationship is not straightforward.[36]

The actual recorded plot for micellar solutions containing (a) 1-methyl-4-*n*-dodecylpyridinium iodide(aq; 303 K)[37] (**I**), (b) CTAB(aq; 298 K)[38] (**II**) and (c) both 1-ethyl- (**III**) and 1-propyl-4-*n*-dodecylpyridinium iodide(aq; 303 K)[39] (**IV**)

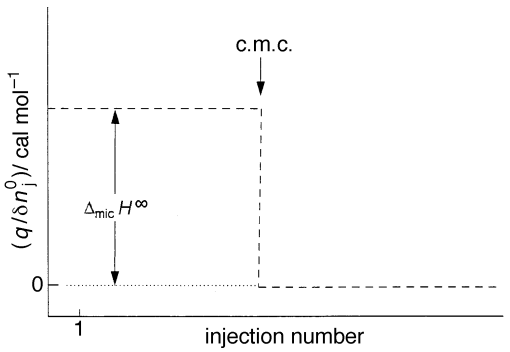
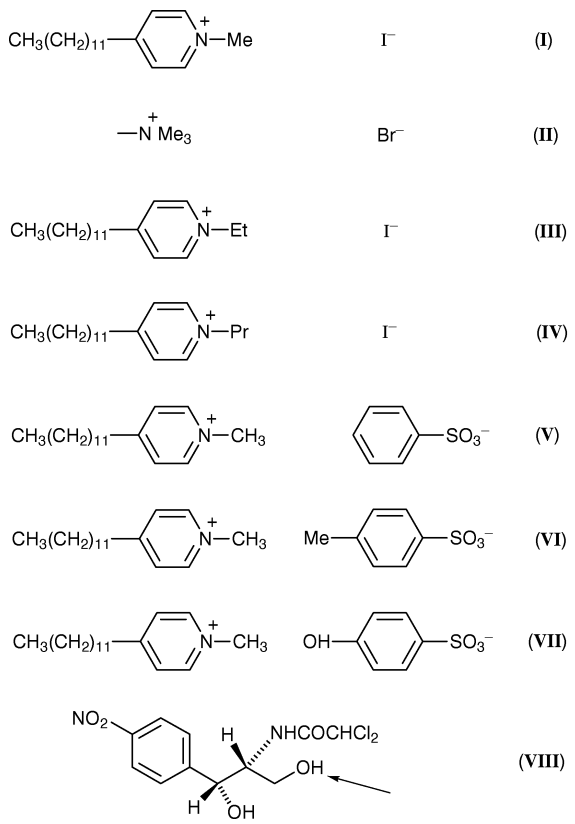


Fig. 4 Idealised plot showing dependence of $(q/\delta n_j^0)$ on injection number for ITC experiment involving injection of an aliquot of an ionic micellar solution into a sample cell containing initially water.



produce enthalpograms which closely resemble the idealised form shown in Fig. 4 and 5.

Several important generalisations emerge from these and related studies. For example, the limiting enthalpy of micelle formation becomes more exothermic[37] by -2.6 kJ mol^{-1} per CH_2 group for 1-methyl-4-alkylpyridinium iodides. With increase in hydrophobicity of counter-ions (*e.g.* changing from **V** to **VI**) the enthalpy of micelle formation becomes slightly more exothermic, -9.0 to -10.0 kJ (mol monomer)$^{-1}$ at 303 K. Introduction of an OH-group in the *para* position of the benzene ring in the counter-anion (*e.g.* **V** to **VII**) also leads to a more exothermic enthalpy of micelle formation. These comments indicate the level of detail which these ITC-based studies yield concerning micelle formation by ionic surfactants in aqueous solutions. In addition, by recording these titration curves at several temperatures, the dependence on temperature of $\Delta_{mic}H^{\infty}$(aq) for a given surfactant yields the corresponding isobaric heat capacity quantity,[40] $\Delta_{mic}C_p^{\infty}$(aq). For example,[39] $\Delta_{mic}C_p^{\infty}$(aq) for **I** is -449 J K^{-1} (mol monomer)$^{-1}$.

Two important applications of ITC in the present context are noteworthy. The first concerns the study of mixed surfactant systems[41] in which the injected aliquots contain a mixture of two surfactants in a known molar ratio. The second group of applications[42] concerns solubilisation by micelles of apolar solutes in which aliquots contain, for example, an aqueous solution comprising CTAB and *n*-pentanol. When the micelles break up, the pentanol is released into the aqueous solution. Then as the concentration of CTAB exceeds the c.m.c. in the sample cell, pentanol is re-solubilised into the micelles.

The foregoing comments centre on those ionic surfactants which produce enthalpograms following the pattern shown in Fig. 5. With decrease in hydrophobicity of the surfactant the magnitude of the enthalpy of micelle formation decreases and

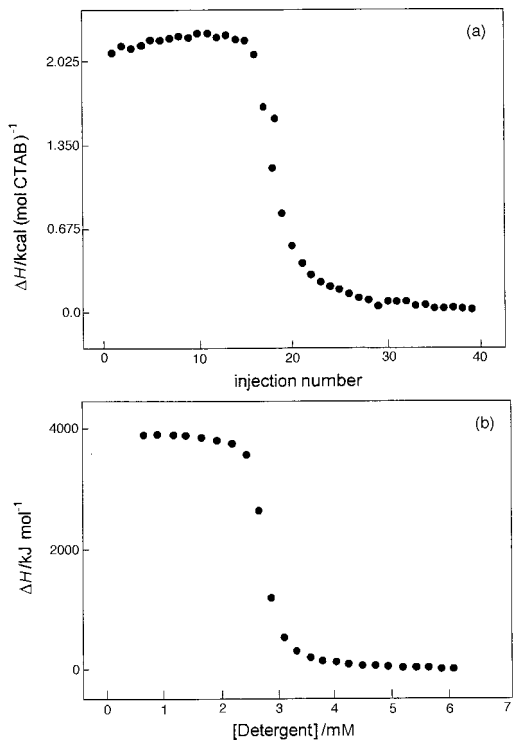


Fig. 5 (a) ITC recorded plot for injection of aliquots (5×10^{-6} dm^3) of CTAB (7.77×10^{-8} mol at 1.54×10^{-2} mol dm^{-3}) into a sample cell (1.411 cm^3) containing water at 298.2 K; c.m.c. = 0.95 mol dm^{-3}, $\Delta_{mic}H^{\infty}$ (aq) = -10.2 kJ mol^{-1}. (b) ITC recorded plot for injection of aliquots (10×10^{-6} dm^3) of ionic surfactant 4-dodecyl-1-methylpyridinium iodide (**I**) (74.2×10^{-3} mol dm^{-3}) into a sample cell volume (1.3 cm^3) containing water at 303 K; c.m.c. = 2.50×10^{-3} mol dm^{-3} and $\Delta_{mic}H^{\infty}$(aq) = -17.9 kJ mol^{-1}.

the c.m.c. increases. The latter means that the concentrations of surfactant in aliquots and in the sample cell are higher in order that the enthalpogram scans from below to above the c.m.c. Consequently, the enthalpograms become somewhat more complicated,[43] reflecting the fact that the systems under investigation are reasonably concentrated salt solutions. In other words, the properties of these solutions are determined in part by contributions from strong and long-range ion–ion interactions.

For surfactants such as tetradecyltrimethylammonium bromide(aq), the injections become gradually more endothermic with increase in injection number[43] and the c.m.c. is less sharply defined. The overall pattern can be accounted for by taking into account the non-ideal properties of the solutions. The pattern was identified[43] as Type B. The anionic surfactant sodium dodecylsulfate is Type B at 298.2 K where a maximum in the enthalpogram[44] is close to the literature[45] c.m.c. value 8×10^{-3} mol dm^{-3} in aqueous solution. The c.m.c.s for the corresponding nickel, cadmium and copper dodecylsulfates in aqueous solution have been similarly determined using a titration microcalorimeter,[46] confirming the values published by Bury and Treiner.[47]

With further decrease in hydrophobic character, the c.m.c. increases markedly and the magnitude of the enthalpy changes are much smaller. Consequently, the enthalpograms, classed[43] as Type C, have a quite complicated shape for which the analysis is certainly not straightforward.[43,48]

6 Protein–ligand interactions

In an important application of titration microcalorimetry,[3–5] the sample cell contains an aqueous solution of an enzyme E and the syringe contains a solution of the substrate S. The composition of the sample following injection of an aliquot of substrate solution is described using the following chemical equilibrium where the extent of chemical reaction is ξ for a system prepared in volume V containing n_S^0 and n_E^0 moles of substrate and enzyme respectively.

$$\mathrm{E(aq)} + \mathrm{S(aq)} \rightleftharpoons \mathrm{ES(aq)} \qquad \text{(I)}$$

If the thermodynamic properties of the solution are ideal, the equilibrium composition of the solution is described by a binding equilibrium constant (concentration scale), eqn. (14).

$$K_B = \xi V/[(n_E^0 - \xi)(n_S^0 - \xi)] \qquad (14)$$

As written, eqn. (14) describes association. It should be noted that many biochemists describe the equilibrium (I) in terms of a dissociation equilibrium constant $K_D(=1/K_B)$ to reflect the stability of the ES-complex. A common assumption is that the solutions are dilute and have ideal properties so that $(\partial H/\partial \xi)_{T,p}$ in eqn. (4) can be replaced by the limiting enthalpy of binding, $\Delta_B H^\infty$(aq). The key quantity in the analysis of the enthalpogram is the dependence of (equilibrium) extent of binding, ξ, on the total amount of substrate n_S^0 in the sample cell. With increase in injection number I (and hence n_S^0), a smaller proportion of injected substrate is bound by the enzyme. In the limit of large K_B (*i.e.* tight binding), all injected substrate is bound until all binding sites are occupied. Hence the recorded heat drops rapidly to effectively zero after remaining constant for the initial set of injections. With decrease in K, the shapes of the enthalpogram change significantly (see below). In this series of experiments it is important to determine the contribution to the recorded heat from simple dilution of the injected aliquot (*i.e.* in the absence of enzyme). This contribution is subtracted from the recorded enthalpogram when the sample cell contains the enzyme solution.

In the analysis of experimental data the key quantity is ξ. Eqn. (14) is written as a quadratic in ξ for which only one solution has physical significance. The equation for ξ in terms of K_B, V, n_S^0 and n_E^0 is differentiated to yield $d\xi/dn_S^0$, eqn. (4). In detail, the magnitude of K determines the shape of the enthalpogram. The analysis[3] is described in detail in ref. 5. A key quantity C is defined, eqn. (15), by the product of K_B and the total concentration of enzyme in the sample cell, see Fig. 3 in ref. 3.

$$C = K_B[E - \text{total}] \qquad (15)$$

In the limit C is infinitely large (*e.g.* $\geqslant 1000$), the enthalpogram is step-shaped, the break occurring when (*cf.* reaction I) n_x^0/n_m^0 is unity. The ratio q/dn_x^0 is constant over the injections up to this point, directly yielding $\Delta_B H^\infty$(aq).

For systems where $40 \leqslant C \leqslant 500$, the enthalpograms are sigmoidal in shape. The intercept on the q/dn_x^0 axis yields $\Delta_B H^\infty$(aq) and the dependence on injection number yields the binding equilibrium constant. An example[49] concerns the binding of chloramphenicol (**VII**) to the enzyme chloramphenicol acetyltransferase (CAT). Chloramphenicol (CMP) was one of the first broad spectrum antibiotics[50] but its efficacy was diminished by an acetyl CoA dependent acetylation of the C(3) hydroxy group (see arrow in structure **VIII**) in which the enzyme CAT(III) is directly involved.[51] The structure of CAT(III) in its active form is trimeric; the active sites are located at the subunit interfaces.[52] The ITC results produce the binding curve shown in Fig. 6 where the measured dependence has been fitted using the previously reported[53] binding equilibrium constant, 2.32×10^5 dm^3 mol^{-1} where $\Delta_B H^\infty$(aq) is -41.4 kJ mol^{-1}.

The ITC technique is now part of the armoury of biophysical chemists. A few examples support this view. Examples where the titration curves show the complete sigmoidal curve are as follows.

(a) Interaction of (injected) Ac-Lys-Gly-Gly-Gln-Tyr(P)-Glu-Glu-Ile-NH_2 phosphopeptide(aq) with (in the sample cell) {glutathione S transferase}–{Src homology 2} fusion protein.[54]

(b) Binding of (injected) *N*-acetyl-Lys-D-Ala-D-Ala peptide ligand to vancomycin.[55] (This system actually involves a peptide antibiotic interacting with its target peptide sequence rather than a typical enzyme–substrate interaction.)

(c) Binding[56] of epidermal growth factor (EGF) to the extracellular domain of its receptor, sEGFR.

With a decrease in the quantity C, eqn. (15), so the titration curve displays less of the sigmoidal curve because only a fraction of injected substance X binds to the enzyme. Consequently, the curve fitting exercise becomes extremely important from a statistical standpoint if reliable estimates of the binding equilibrium constant and binding enthalpy are to be obtained. The following studies illustrate this point.

(a) Binding of calcium ions to a mesophilic xylanase.[57]

(b) Binding[58] of ATP to wild-type phosphoglycerate kinase (PGK).

(c) Binding of ammonium dehydroquinate to type II dehydroquinases.[59]

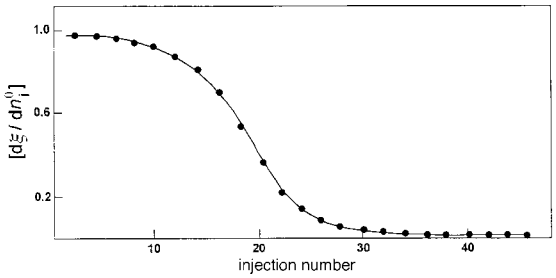


Fig. 6 ITC results for injection of aliquots of CMP (aq; 8×10^{-3} mol dm^{-3}; 2×10^{-6} dm^3) into CAT (**III**) (aq; 0.2×10^{-6} mol dm^{-3}; volume = 1.4 cm^3) at 298 K

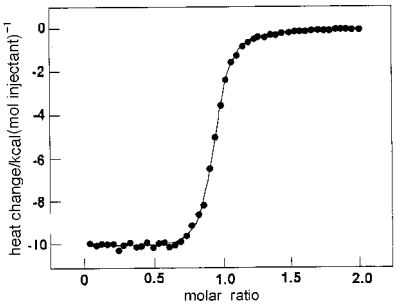


Fig. 7 ITC enthalpogram for titration of aliquots of 1-adamantane carboxylic acid (aq; 0.53×10^{-3} mol dm^{-3}) into β-cyclodextrin (aq; pH 4.00; 0.1 mol dm^{-3}); calculated $K_B = 4.46 \times 10^6$ dm^3 mol^{-1} and $\Delta_B H^\infty(\text{aq}) = -41.8$ kJ mol^{-1}

(d) Binding[60] of tyrosine phosphorylated proteins to SH2 domain of p56^{lck}.

In some instances there is more than one binding site in the enzyme and so the number of binding sites, n, is a variable in the curve fitting[61–64] in addition to K_B and $\Delta_B H^\infty$. An interesting development concerns the development of equations describing enthalpograms produced by consecutive binding equilibria[65] and by dissociation of macromolecules, *e.g.* insulin.[66]

7 Guest–host complex formation

Application of ITC techniques in the subject area[67–70] considered in this section takes its lead from the subject matter and data analytical methods described in the previous section. A typical example[71,72] is shown by the titration plot for injection of 1-adamantane carboxylic acid(aq) into β-cyclodextrin in aqueous buffered solution, Fig. 7.[72] Danil de Namor and coworkers have used a titration microcalorimeter (sample volume 2.8 cm^3 with injected solution from 0.5 cm^3 gas-tight Hamilton syringe, injected aliquots at 5 min intervals) in an extensive study of alkali-metal binding in non-aqueous solvents,[73–75] *e.g.* sodium ion with methyl *p-tert*-butylcalix[4] arenetetraethanoate in cyanomethane at 298.2 K.[73]

8 Adsorption

The phenomenon of binding of a substrate S to an enzyme E (Section 6) could be described as adsorption of a small molecule by a macromolecule in which the site of adsorption is uniquely defined. In the more general case, we might consider a water soluble polymer [*e.g.* poly(*N*-vinylpyrrolidone), PVP] which provides a surface, the adsorbent, on which the adsorbate is adsorbed. (Terminology is important here. In the latter subject, the term 'substrate' has the same meaning as absorbent, being the host. In enzyme chemistry, the term 'substrate' is the guest which binds to the host.) The phenomena described in this section are broadly analysed[44] following the methodology described by Langmuir[76] for adsorption of gases on solids; the procedure is based on opposing rate processes for adsorption and deadsorption. However, the phenomenon is also described in terms of a thermodynamic equilibrium for chemical substance-j either adsorbed or free in solution. The chemical potential of the adsorbed substance-j is related[44] to the fraction of the surface area covered, θ, [eqn. (16), *cf.* ref. 77 and 78] using eqn. (16).

$$\mu_j(\text{ads}) = \mu_j^0(\text{ads}) + RT \ln[\theta/(1-\theta)] - RT\phi\theta \qquad (16)$$

The quantity μ_j^0(ads) is the chemical potential of the adsorbed substance when θ equals 0.5 and in an ideal system where there are no interactions between the adsorbed molecules on the adsorbent. The quantity ξ [*cf.* eqn. (1)] describes the extent to which substance-j is adsorbed. If the adsorption is described by an equilibrium constant K then in a solution volume V containing, in total, n_j^0 moles of substance-j

$$K = [\pi\xi/(1-\pi\xi)]Vc_r/(n_j^0 - \xi) \qquad (17)$$

Here π is characteristic of the total area of adsorbent and the cross-sectional area of the adsorbate. The similarity with eqn. (14) is clear in that both yield quadratic equations for ξ. The analysis is developed to yield the required form of eqn. (4) taking into account a term describing the dependence of enthalpy of adsorption on θ [*cf.* the third term in eqn. (16) involving the product $\phi\theta$]. The enthalpogram describing the adsorption of SDS micelles(aq) on PVP(aq) can be accounted for using this approach,[44] Fig. 8.

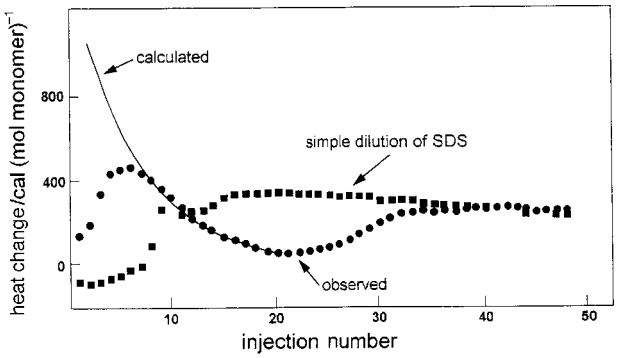


Fig. 8 Enthalpogram[44] describing the adsorption of SDS micelles (aq; 800×10^{-3} mol dm^{-3}; 5×10^{-6} dm^3 injections) into sample cell containing PVP (aq; 1%; 1.4 cm^3); the full line is calculated using equations based on eqn. (16) and (17)

A similar pattern is observed for adsorption of sodium decylsulfate[44] and related[45] copper and cadmium surfactants on PVP(aq). We suggest that, based on the shape of the enthalpograms, a similar model might account for the binding of disubstituted anthracene-9,10-diones to duplex and triplex DNA[79] and of a cyclic peptide to lipid membranes.[80]

9 Summary

The review has attempted to show the versatility of the isothermal titration microcalorimeter (ITC) in several key subject areas. We have shown that the technique relies on two key statements in thermodynamics concerning the definition of equilibrium for closed systems held at fixed temperature and pressure and concerning the identification of the heat passing between system and surroundings at constant pressure as the change in enthalpy. These statements lead, for example, to information concerning equilibrium constants and enthalpies of reaction.[81]

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